FURTHER EXPERIMENTS ON THE METHODS OF EGG-LAYING IN AMPHITRITE.

JOHN W. SCOTT.

INTRODUCTION.

On excursions, taken in connection with the course offered to students studying invertebrate zoölogy at Woods Hole, I have been impressed with the large number of forms not used for investigation. I believe this must be because little is known of their life-history and habits, because many of them are fairly abundant. With this idea in view, I recorded in a recent paper ('09) the results of some observations made upon the egg-laying habits of one of the marine annelids. In the form studied, *Amphitrite*, the germ cells arise in the typical way, *i. e.*, from the cœlomic epithelium. Very early these dehisce into the body cavity and mix with the cœlomic corpuscles. In some annelids as *Nereis*, the eggs escape by a rupture of the body wall. In *Amphitrite*, however, they pass to the exterior through certain nephridia that are highly modified to form gonaducts. In the paper mentioned above it was shown that *Amphitrite* deposit eggs at recurring periods that bear a close relation to the time of spring tides. It was further shown that at the time of oviposition, the body cavity contains floating free, not only the mature eggs and corpuscles but also the younger eggs in various stages of development. A single period of egg-laying occupies from 30–60 minutes, and most all eggs when extruded are in the metaphase of the first polar spindle. A few large, though unripe, eggs always escape especially toward the latter part of the period. In Fig. 1 is shown the comparative size and shape of various bodies found in the cœlome at this time. It will be noticed that the eggs when first set free in the cœlome are smaller than the red blood cells, yet neither cells nor eggs of this size ever escape during oviposition. Indeed, comparatively few of the large unripe eggs pass out through the nephridia. The

interesting question then arises, *How are ripe eggs separated from the other cœlomic bodies in the act of oviposition?*

REFERENCES TO PREVIOUS PAPER.

At first an attempt was made to answer this question by studying the general anatomy of *Amphitrite*. In this group, as shown by Meyer, the septa are incomplete or absent, with one exception. The one complete septum, called the diaphragm, is near the anterior end and divides the cœlome into two unequal cavities. Anterior to the diaphragm the nephridia are for ex-
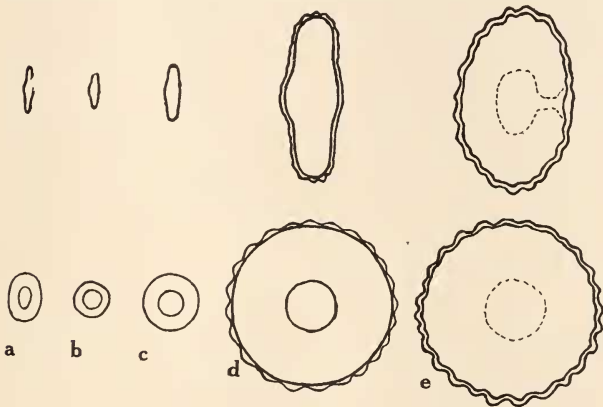

FIG. 1. To show the comparative size and shape of various bodies found in the cœlome of *Amphitrite* at the time of egg-laying; *a*, red blood corpuscles; *b*, youngest free egg observed; *c*, young egg, with little or no yolk; *d*, unripe egg, nearly mature; *e*, mature egg. Both edge and side views shown.

cretion only. The posterior nephridia are modified as gonaducts, and it is with these last that we are concerned. Their inner openings are bordered by large folded, or fimbriated, membranes, covered by strong cilia. Each opening connects with a large vascular sac, also ciliated. From these sacs tubes pass to the outer openings of the nephridia which are found in the species studied on segments 6–10 inclusive. From the dissections I came to the conclusion that there was no apparatus for sifting or "straining out" the ripe eggs.

However a theoretical explanation was given in which the nephridia were regarded as "settling basins," the cilia in this case preventing the settling of bodies which were not to be expelled. This explanation was based partly upon the structure of the nephridia, and partly upon the fact that "when the contents of the cœlome are stirred in sea-water, the largest ova including the ripe eggs, always settle more quickly to the bottom of the dish; the immature eggs then settle and last of all the cœlomic corpuscles." The important fact here is that gravity more quickly influences the large ova than the other bodies floating in the cœlomic fluid. It was suggested that the difference in the rate of settling was probably due to a greater density of the ripe eggs, but that the effect was possibly due to a difference in the shape of the bodies concerned. In either case, the conclusion was drawn that gravity is a differential means of separation. However, this left the matter inconclusive and the question arose, *Is this tendency of large ova to settle quickly due to a greater specific gravity, or to a greater mass in proportion to the amount of surface offering resistance?* To answer this was the primary object in the next step of the investigation.

Specific Gravity Experiments.

Having set the problem, I began the work of determining the specific gravity of the eggs and corpuscles found in the fluid of the body cavity. Incidentally the specific gravities of sperm, and of eggs in some later stages of development were obtained.

Lyon's plan of getting the density of eggs by centrifuging in gum arabic solution was tried and, after finding it suited my purpose, his method was adopted with slight modifications. First a strong gum arabic solution in sea-water was prepared and its density carefully determined. From this stock solution a series of stock solutions was made up of differing densities; this can be readily done by mixing with the stock solution in proper proportions sea-water of known density. When ready to centrifuge the capillary tubes of a hæmatocrit were filled about three fourths full of gum arabic solution. On top of these solutions whose density was known was placed the material whose density was required. In a few cases, in order to guard

against possible error caused by surface tension, the material was placed between two solutions of differing densities and then centrifuged. But surface tension did not appear to offer any appreciable resistance to the passage of the eggs and corpuscles either upward or downward. It was found that eighty turns of the hæmatocrit in one and one half minutes was sufficient to produce thorough separation of materials in the capillary tubes, without noticeably affecting the material within the egg membrane. This amount of centrifuging was therefore adopted throughout the entire series. I shall here present in order my findings in regard to (1) the specific gravity of eggs, (2) the specific gravity of corpuscles, and (3) the specific gravity of sperm, together with remarks as to the significance of these results.

After a number of preliminary experiments, in order to get familiar with the method to be used and to guard against possible sources of error, a series of tests was made upon eggs recently deposited. The results of some of these tests are shown in Table I. The female used in this series was found depositing

TABLE I.

To Show the Specific Gravity of Recently Deposited *Amphitrite* Eggs

Test No.	Density of Solution Used.	Material Used.	Result of Centrifuging.
25	1.090	Eggs just deposited.	Eggs on top.
26	1.085	Eggs just deposited.	Eggs on top.
27	1.080	Eggs just deposited.	A few begin to sink.
28	1.075	Eggs just deposited.	All went down.
29	1.0775	Eggs just deposited.	More than one half sink.
30	1.075	Eggs just deposited.	All on bottom.

eggs in a normal manner. Just as quickly as it could be done, while they were still being deposited, these eggs were centrifuged with the results shown in tests 25 to 30 inclusive. The table shows that the specific gravity of recently deposited, that is, mature *Amphitrite* eggs, lies between 1.075 and 1.085. They probably have a mean density very near 1.078; for almost all eggs remain on top of, and therefore are lighter than a solution with a specific gravity of 1.080, while more than half sink in a solution with density at 1.0775. Tests were made on eggs from other worms and verified the results given here.

Another series of tests was made in order to demonstrate the changes in density at different stages of development. See Table II. It was shown that very young eggs, those that had

TABLE II.

To Show the Change in Density at Different Stages in the Development of Eggs.

Test No.	Density of Solution Used.	Material Used.	Results of Centrifuging.
9	1.070	Cœlomic contents. Eggs half-grown and smaller.	Youngest eggs on top.
12	1 075	Cœlomic contents. Eggs nearly mature.	All eggs went down.
13	1.075	Eggs in various stages.	Unripe eggs on top. Ripe eggs down.
27	1.080	Mature eggs, just deposited.	Almost all on top, a few sink.
38	1.080	Same lot, after being in sea-water 1½ hours.	One half of eggs sink.
41	1 080	Same lot, fertilized. 8–16-celled stages.	One fifth of eggs sink.
47	1.075	Same lot, one hour later.	A few eggs sink. Most eggs partly down.
57	1.075	Same lot, trochophores, age 27 hours.	One third trochophores sink Best developed on top.

attained one fourth the size of mature eggs and in which there was not very much yolk laid down, had a specific gravity less than 1.070 (Fig. 1, c). In a word, the density of the egg as a whole is very noticeably increased as the yolk accumulates. It was also learned that allowing the deposited eggs to stand in sea-water for some time slightly increases their density (test 38). During segmentation and the formation of the segmentation cavity, the density grew less as one might expect (tests 41, 47). It probably continues to decrease slightly, at least as far as the late trochophore stage (test 57). My experiments did not carry the work further than this point.

Before the worm used in the first tests mentioned above, was through depositing eggs, the surface of its body was wiped dry and the cœlome was opened allowing the contents to escape into a clean glass dish. An examination with the microscope showed that the cœlomic fluid contained corpuscles and eggs in various stages of development, some being mature. Tests 32 to 37, Table III., show typical results in regard to the specific gravity of the corpuscles. Upon examining these results one finds that

TABLE III.

To Show the Specific Gravity of Cœlomic Corpuscles.

The cœlomic contents were removed after the worm was about through depositing eggs.

Test No.	Density of Solution Used.	Material Used.	Result of Centrifuging.
32	1.080	Cœlomic corpuscles. Eggs in various stages.	Corpuscles sink. A few ripe eggs sink.
33	1.085	Cœlomic corpuscles. Eggs in various stages.	All corpuscles sink. All eggs on top.
34	1.090	Cœlomic corpuscles. Eggs in various stages.	Seven eighths corpuscles down. Eggs on top.
35	1.095	Cœlomic corpuscles. Eggs in various stages.	Most corpuscles sink. Eggs on top.
36	1.105	Cœlomic corpuscles. Eggs in various stages.	Two thirds corpuscles sink. Eggs on top.
37	1.123	Cœlomic corpuscles. Eggs in various stages.	One tenth corpuscles sink.
46	1.123	After standing three hours in sea-water.	Nearly one third corpuscles sink.

the specific gravity of the cœlomic corpuscles varies between wide limits. However, more than four fifths of them have a density greater than 1.090 and less than 1.123. Contrary to what my previous observations had led me to expect the corpuscles have a density greater than the mature eggs. All corpuscles are heavier and all eggs lighter than a density of 1.085. After standing in sea-water for some time the corpuscles appear to increase in density as shown by test 46, and by other tests not given. Furthermore, the smaller and what appear to be the younger corpuscles have a density less than the older ones. Under these circumstances an examination with the microscope after centrifuging was of course necessary to distinguish between the corpuscles. The significance of these results will be explained later.

In the course of my experiments the cœlomic corpuscles of both males and females were examined. While not suspected at the time, upon looking over my notes the rather curious fact came to light that the mean density of the female corpuscles is slightly greater than that of male corpuscles. I do not wish to emphasize this fact, for perhaps the number of individuals examined was not sufficient on which to base conclusions. Nor yet do I see whether the significance of these results pertains to nutrition

alone or to differences in reproduction, should they prove gener-
ally true for other annelids.

Another series of experiments showed the extreme lightness of
the sperm; their specific gravity in sea-water being between
1.038 and 1.046. In contrast to eggs which increase in density
as they mature, the sperm masses decrease in density as they
grow larger and finally break up into free-swimming sperm. So
that the density of the ripest sperm is even less than 1.038, as
shown in Table IV. This density is interesting when considered

TABLE IV.

To Show the Specific Gravity of Sperm.

Test No.	Density of Solution Used.	Material Used.	Result of Centrifuging.
8	1.075	Contents of cœlome.	All sperm on top. All corpuscles sink.
40	1.075	Sperm shed in sea-water.	All sperm on top.
42	1.070	Sperm shed in sea-water.	All sperm on top.
43	1.062	Sperm shed in sea-water.	All sperm on top.
44	1.051	Sperm shed in sea-water.	All sperm on top.
45	1.038	Sperm shed in sea-water.	All sperm at bottom.
48	1.046	Sperm shed in sea-water.	All sperm on top.
50	1.041	Cœlomic contents of worm that deposited sperm on previous day.	All unripe sperm sink; a few nearly ripe on top.
52	1.046	Cœlomic contents of worm that deposited sperm on previous day.	Younger sperm masses sink. Older sperm masses on top.
55	1.051	Cœlomic contents of worm that deposited sperm on previous day.	Younger sperm masses sink.
56	1.038	Cœlomic contents of worm that deposited sperm on previous day.	A few lightest, ripest sperm on top.

in connection with fertilization and the habitat in which the
animal lives. Water currents are undoubtedly important factors
in the dissemination of sperm. But the sand flats on which
these animals are found are securely protected from violent cur-
rents, where reefs or eel grass or shoals of adjacent islands break
the force of the changing tide. The limited range of the animal
does not require a wide scattering of the sperm. In fact the
density of the sperm is such that, in the presence of very gentle
currents, their distribution must be very limited at the time of
oviposition. But the lightness of the sperm provides an easy

means for scattering them by currents over the sand flats, and their own locomotion aids them in their distribution. They have sufficient motor power to aid in horizontal distribution, and have sufficient density to prevent them from rising much above the bottom where the eggs are found. That sperm have such a method of distribution was substantiated in a different way by studying the scattering of sperm in dishes of still water. A large quantity of sperm was introduced at some point in the bottom of the dish; then the progress in distribution was observed by noting the advancing cloudiness of the water, reckoned in various directions from the point of departure. It was found that the advance in a general horizontal direction was many times more rapid than the advance in an upward direction. I take this to be due to the resistance of gravity. Since the sperm are set free near the bottom under normal conditions, their lightness tends to bring about more favorable chances for fertilization of the eggs.

We may sum up the results of these specific gravity experiments in the form of certain explanations and conclusions:

1. The eggs of *Amphitrite* increase in specific gravity during growth in the cœlome. This is probably due to an increase in the amount of yolk. The reason therefore that the larger mature eggs settle in a dish of sea-water more rapidly than the smaller, immature ones, is undoubtedly due in part to the greater specific gravity of the older eggs. And in the process of egg-laying it is probable that a difference in specific density may act as a means to separate ripe from unripe eggs.

2. The reason why the larger eggs sink before the cœlomic corpuscles must be explained in another way, for the specific gravity of the corpuscles is decidedly greater than that of the eggs. The difference in behavior between these bodies is to be explained, I believe, by the flat, oblong shape of the corpuscles; their shape is such that they offer in settling a much larger resistance in proportion to their mass. Blood cells settle more slowly than eggs in sea-water because of a difference in shape and not because of a lesser density. Slight currents in the dish prevent the corpuscles from settling for a long time, while they hardly interfere with the downward movement of the eggs. At this

time in my experiments it was thought probable that ciliary action produces a similar effect in separating eggs and corpuscles during oviposition. This will be discussed later.

3. While the egg increases in density up to the time of oviposition it decreases slightly after fertilization, the decrease continuing at least as far as the trochophore stage.

4. The comparative lightness of the sperm is undoubtedly useful as an adaptation for scattering them at the time of fertilization. Their density is just sufficient to tend to keep them near where the eggs are to be found, but not enough to prevent a certain amount of locomotion.

Direct Observations.

During the past summer I was able to make a series of direct observations upon the separating process. In these observations I saw the ripe eggs pass over the fimbriated membrane, along the grooves, and finally into the vascular, nephridial sac ready to be expelled. At the same time the blood cells and unripe eggs were rejected and thrown back into the cœlomic fluid. The sight was indeed astonishing to see the general precision of a comparatively simple process.

It was by means of living dissections that the observations were made possible. First it is necessary to use only ripe females, and to determine this fact with certainty one needed to wait until the worm began to deposit eggs; then it was lifted out of sea-water and the surface of the body quickly dried. Next the cœlome was opened and its contents carefully drained into a clean watch glass; this was kept covered to prevent unnecessary evaporation while the nephridia were being removed. The worm was then placed in a dry dissecting pan and pinned at either end, so that it was held in a slightly stretched condition. The body cavity was opened by making a cut through the body wall in the mid-dorsal line; this cut began a little back of the middle of the body and extended forward to the anterior end. The enteric canal and accompanying blood vessels were next divided midway and their anterior portions removed. The flaps of the body wall on either side were then pinned out. In order to have these flaps spread out well, and so expose the large

nephridial sacs, it was necessary to cut the series of oblique muscles which extend from the body wall above the notopodial cirrus across the cavity to the body wall in the mid-ventral region. This species has five pairs of nephridia that function as oviducts. The cœlomic fluid of a ripe female contains some ripe eggs, many unripe ones, and thousands of corpuscles, mostly red blood cells. Each nephridium to be observed was removed entire and placed at once in the watch glass with the cœlomic fluid. Then the watch glass was placed on the stage of a microscope and the process studied.

As mentioned before the fimbriated membrane is a folded, or grooved structure covered for the most part with strongly developed cilia. The stroke of the cilia varies in direction and force on different parts of the membrane and their action furnishes the motor power that separates the eggs. By using a needle I could push near the fimbriated membrane any of the bodies I wished to study. The cilia are in continuous action, and their effective stroke, so far as I could observe, has a constant general direction. But the direction of the stroke varies on different portions of the membrane as follows: (1) On the edge of the membrane (Fig. 2, a) and near the entrance to the grooves between the folds, the stroke is such as to send currents toward or into the grooves. (2) In the grooves (Fig. 2, b), where the folds approach somewhat nearer to each other, the stroke at the bottom of the groove is still onward and inward; well up on the sides of the grooves the currents move upward and outward, and apparently with more force than at the bottom of the groove. (3) In the main groove (Fig. 2, c) the cilia at the bottom are arranged in approximately parallel lines that resemble small grooves, all leading inward and their action comparatively slow. Above on the sides of the pillars (Fig. 2, d), especially in the narrow grooves between pillars, the currents move rapidly and are directed upward and outward. With this explanation of the ciliary currents, both in regard to position and action, we may now consider how the different bodies are separated.

The Separation of Blood Cells from Eggs.—This occurs under the conditions of my experiment principally on the outer edges of the membrane. The currents are strong enough to lift and

draw in toward the membrane blood cells from a considerable distance; these cells therefore hit the cilia with some force, but the major stroke of the cilia is strong enough to bounce them off again, usually driving them away five to ten times their own diameter, and frequently beyond the limits of further attraction. However they may bounce two, three, or four times, that is,
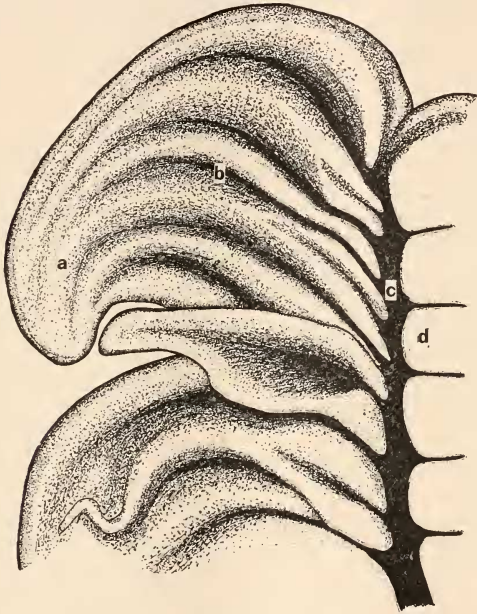

FIG. 2. To show a portion of the fimbriated membrane found at the inner end of a post-diaphragmatic nephridium of *Amphitrite*. Not so much magnified as Fig. 1. The membrane is undulating at *a*, and folding to form a groove at *b*; at *c* is the deep main groove leading into the nephridial sac; at *d*, are pillar-like projections overhanging the main groove. For further explanation see text.

until they reach the region of the groove shown at *b*, Fig. 2; then the cilia on the sides carry them up, out, and away. Only in very rare instances do blood cells ever reach the main groove; sometimes they follow the eddy near a mature egg, but they are

then thrown across the groove and against the pillars where the currents hurriedly lift them up and drive them away. I did not see a blood corpuscle pass down the main groove into the nephridial pouches, so long as the cilia retained their normal activity. After an hour or so their action becomes slower, but this is probably due chiefly to the fact that much plasma has evaporated.

The Separation of Immature from Mature Eggs.—The small, immature eggs, Fig. 1, *b* and *c*, as well as some of a larger size are eliminated in much the same way as blood cells. The task grows more difficult, however, as the eggs increase in size for they become too bulky to be bounced off the edge of the membrane like the blood cells. So, as the larger immature eggs (Fig. 1, *d*) are carried into the narrowing grooves, they are rolled up on edge, then lifted out and moved away. For when the sides of the groove narrow down so that both sides of the flat egg are affected by the currents produced by cilia on the opposite sides of the groove, the combined action of these currents is sufficient to lift even the weight of a large, immature egg. The case is different with the mature eggs, which *differ in shape*, are *more plastic*, and are *somewhat heavier* than most immature eggs found in the cœlome. These eggs are carried into the grooves down which they slowly pass, their shape and weight evidently being such that the lateral cilia are unable to lift them out of the groove. Undoubtedly their plasticity is also an important factor in resisting the action of the cilia. The side of the egg gives before the effective stroke of the cilia, so the result is to change the shape of the ripe egg rather than to push it away, and consequently such eggs move more slowly along the grooves. In some cases it was observed that the folds of membrane along the sides of the groove would respond to the presence of a ripe egg by folding over, virtually forming a closed tube. Inside this tube-like groove the egg could occasionally be seen changing shape, as it was forced slowly along toward the nephridial sac. When the membrane folded it was impossible for unripe eggs, or corpuscles on the membrane, to pass into the nephridial pouch with the ripe egg. The behavior of the membrane in the presence of a ripe egg had all the appearance of a tactile response.

and if any chemical reaction was here involved it could not be detected with the eye. The separation of these bodies appeared to be a purely physical process.

Since the cilia are continuously active, independent of any egg-laying period, it would seem that the egg-laying reaction is a direct response to changes in the egg that are chiefly produced by the breaking down of the germinal vesicle. As noted in an earlier paper a worm shows unusual muscular activity at the time of oviposition. This muscular reaction may be due to chemical changes in the body fluid, the irritation being produced by materials escaping from the nucleus. However another explanation is possible. As the ripened eggs begin to accumulate in the nephridial sacs, excretion, the normal function of these organs, would be hindered or stopped. This would lead to an accumulation of waste in the part of the cœlome posterior to the diaphragm, and in all probability would act as an irritant producing more vigorous muscular action until the eggs were expelled. In this way the wave-like movements of the worm at oviposition are a physiological response to an interference with excretion. Though these suggestions are largely theoretical, I am inclined to believe the latter view is the correct explanation.

Summary and Conclusions.

Amphitrite under normal conditions keeps up, with occasional brief resting periods, a series of wave-like contractions of the body for the purpose of sending water through its tube. A short time before oviposition the wave-like movements become stronger and slightly faster than usual. This excitement indicates the presence of ripe eggs and marks the beginning of the separating process. After some time has elapsed, and the nephridial sacs are well filled, each contraction wave as it approaches the anterior end of the body forces out through the pores small slimy streams of eggs. Oviposition usually continues from thirty to sixty minutes. The process of separation has been previously described. While the cilia furnish the motor power in separating the ripe eggs, strong wave-like contractions of the body wall furnish the pressure needed to expel the eggs from the nephridial pouches. It is suggested that this unusual activity of the worm

is probably due to interference with excretion, caused by clogging the nephridial sacs with eggs.

Undoubtedly much the same process occurs in other worms where eggs accumulate in nephridial sacs before oviposition. I am told by Downing that eggs accumulate before oviposition in the nephridial pouches of one species of *Arenicola*. Gerould has reported that the eggs of *Phascolosoma* collect in this way for several hours before they are laid, and there is little doubt that the same general method of egg-laying occurs in all worms with this habit. My results may be summarized in the following conclusions.

1. The separation of cœlomic corpuscles and unripe eggs, at the time of oviposition in *Amphitrite*, is accomplished by physical not chemical means.

2. The work of cilia on the fimbriated membrane furnishes the power used in the separating process. Wave-like contractions of the body aid in expelling the eggs, and it is suggested that these movements are due to interference with excretion, caused by clogging the nephridial sacs with ripe eggs.

3. The separating process is aided in several ways. The *shape* and *arrangement of grooves* on the fimbriated membrane, the *size* and *shape* of the bodies separated, and especially the *greater plasticity* and greater *density* of the mature eggs, all appear to be important factors in separating the bodies found in the cœlome at the time of oviposition. The action of the grooves in closing over a ripe egg is also a direct help in keeping other bodies from the main groove.

4. We are fairly safe in concluding that the method of egg-laying described for *Amphitrite* holds good for other worms where the eggs accumulate in nephridial sacs before or during oviposition.